RESEARCH ARTICLE

The first genetic map of pigeon pea based on diversity arrays technology (DArT) markers

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Abstract

With an objective to develop a genetic map in pigeon pea (*Cajanus* spp.), a total of 554 diversity arrays technology (DArT) markers showed polymorphism in a pigeon pea F_2 mapping population of 72 progenies derived from an interspecific cross of ICP 28 (*Cajanus cajan*) and ICPW 94 (*Cajanus scarabaeoides*). Approximately 13% of markers did not conform to expected segregation ratio. The total number of DArT marker loci segregating in Mendelian manner was 405 with 73.1% ($P > 0.001$) of DArT markers having unique segregation patterns. Two groups of genetic maps were generated using DArT markers. While the maternal genetic linkage map had 122 unique DArT maternal marker loci, the paternal genetic linkage map has a total of 172 unique DArT paternal marker loci. The length of these two maps covered 270.0 cM and 451.6 cM, respectively. These are the first genetic linkage maps developed for pigeon pea, and this is the first report of genetic mapping in any grain legume using diversity arrays technology.

Introduction

Genetic mapping is an important subject in biological research and molecular markers, and genetic maps are also important prerequisites for undertaking molecular breeding methodologies for crop improvement. Further in many species, crop improvement programmes have benefited from genetic diversity and mapping studies (Varshney *et al.* 2006). Pigeon pea (*Cajanus cajan* (L.) Mill sp.) ($2n = 22$) is one of the major grain legume crops cultivated mainly by resource-poor farmers in tropical and sub-tropical regions of the world. It is drought tolerant but highly susceptible to some abiotic (water-logging) and biotic (*Fusarium* wilt and sterility mosaic disease, etc.) stresses. Despite past

research efforts, productivity of this crop remained low at around 700–800 kg/ha (http://faostat.fao.org/) over the last five decades. This is mainly due to limited exploitation of available natural variability of genus *Cajanus* in breeding lines.

Although genomics research in pigeon pea has gained momentum recently (Varshney *et al.* 2010), the limited availability of genomics tools in the past has impeded progress in this important crop. For example, to date, there is no linkage map available. To enable a better understanding of the genetic constitution and diversity present in this crop, a set of diversity arrays technology (DArT) markers have been developed. DArT is a dominant marker class which does not have constraints like sequence dependence and high genotyping cost associated with other marker classes

such as simple sequence repeat (SSR), single nucleotide polymorphism (SNP) and expressed sequence tags (ESTs) markers. It is a sequence independent and high throughput technique, which provides practical and cost-effective whole-genome profiling (http://www.diversityarrays.com/). This quality makes DArT a marker of choice for all species regardless of sequence information availability. As the technology is developed using the metagenome, it can cover diversity from cultivated species to wild relatives and can even include diversity surveys of newly developed varieties for analysis. Further, as the same platform is used for discovering and scoring of polymorphic markers, it is cost effective and a user-friendly approach for genotyping of polymorphic markers.

DArT marker system has been developed as a technology for whole-genome profiling in barley (Wenzl *et al.* 2004), cassava (Xia *et al.* 2005), *Arabidopsis thaliana* (Wittenberg *et al.* 2005), bread wheat (Akbari *et al.* 2006), etc. As very few (∼10%) polymorphisms have been identified in cultivated pigeon pea lines using a limited number of SSR and DArT markers (Burns *et al.* 2001; Yang *et al.* 2006), an interspecific mapping population was developed by using pigeon pea (*C. cajan*) accession ICP 28 and ICPW 94 from a wild relative species of pigeon pea (*C. scarabaeoides*) to avail maximum polymorphism and to generate the first generation linkage map. Here we demonstrate that DArTs can be effectively applied to genetic linkage mapping of pigeon pea and report the first genetic linkage map of pigeon pea.

Materials and methods

Mapping population

An accession of cultivated pigeon pea (*C. cajan*) ICP 28 and an accession from wild relative of pigeon pea (*C. scarabaeoides*) ICPW 94 were used as crossing parents for the development of an F_2 population of 72 individuals. DNA from the parents and F_2 progenies were isolated from two to three young leaves following the protocol of Cuc *et al.* (2008).

Library construction

Pigeon pea cultivar (PPC) and wild relative (PPW) libraries were constructed using DNA of parental genotypes ICP 28 for PPC and using DNA from ICPW 94 for PPW through standard DArT procedure using the *Pst*I/*Hae*III library complexity reduction method. This method has been shown to perform best in a previous diversity analysis study conducted in pigeon pea (Yang *et al.* 2006). The pigeon pea Suppression Subtractive Hybridization (SSH) library (Diatchenko *et al.* 1996) was constructed in order to increase the polymorphism of the pigeon pea mapping array. SSH is based primarily on a technique called suppression polymerase chain reaction (PCR) and combines normalization and subtraction

in a single step, which dramatically increases the probability of obtaining low abundance differentially expressed DNAs and simplifies analysis of the subtracted library. The pigeon pea diversity library was combined with a newly constructed libraries of PPC, PPW, and SSH to generate a pigeon pea mapping array for genotyping the F_2 population. A total of 6144 clones in 16, 384-well microtitre plates were used to establish the new pigeon pea mapping arrays (table 1). Both parents and the F_2 population progenies were hybridized to the same mapping array.

Preparation of genomic representations and hybridization to mapping arrays

The mapping samples' genomic representations were generated using the same complexity reduction method (*Pst*I/*Hae*III), which has been developed for construction of the DArT diversity library. Representations were precipitated with 1 volume of isopropanol, denatured at 95°C for 3 min and labelled with fluorescent dye (1.5 μL of 500 μM Cy3-labelled or Cy5-labelled random decamers synthesized by Sigma, Sydney, Australia), using the exo-Klenow fragment of *E. coli* DNA polymerase I (NEB). Labelled representations, called 'targets', were added to 50 μL of a 50 : 5 : 1 mixture of ExpressHyb buffer (Clonetech, Mountain View, USA), 10 gL^{-1} herring sperm DNA, and the Cy5-labelled or FAM-labelled polylinker fragment of the plasmid used for library preparation as a reference (Jaccoud *et al.* 2001). After denaturing, labelled targets were hybridized onto the microarray surface and then covered with a glass coverslip. Hybridization slides were placed in a humidification chamber at 65°C and incubated overnight. After 14 to16 h of incubation, the coverslips were removed; slides were placed in slide-racks and washed in 1× SSC + 0.1% SDS for 5 min; then 1× SSC for 5 min; followed by 0.2× SSC for 2 min and finally 0.02 × SSC for 1 min. Slides were spin-dried immediately at 200 XG (relative centrifugal force), at room temperature for 7 min.

Production and analysis of DArT mapping data

After the slides were dry, they were scanned using a fluorescence microarray scanner (Tecan LS300 scanner, Männedorf,

Table 1. Composition of pigeon pea genetic mapping array.

Library	No. of 384-well plates	Candidate clone	Mapping array clones
(*Pst*I/*Hae*III)G*	5	1920	
(*Pst*I/*Hae*III)PPC	1	384	6144
(*Pst*I/*Hae*III)PPW	5	1920	
SSH	5	1920	

*Selected from previous diversity array (Yang *et al.* 2006).

Switzerland) and images were generated for each of the fluorescent dyes using appropriate laser/filter combinations. The DArTsoft 7, a software package developed in-house (http://www.diversityarrays.com/software.html), was used to automatically analyse each batch of TIF image pairs generated in the experiment. The relative hybridization intensity of each clone on each slide was determined by dividing the hybridization signal in the target channel (genomic representation) by the hybridization signal in the reference channel (polylinker). Clones with variable relative hybridization intensity across slides were subjected to fuzzy k-means clustering to convert relative hybridization intensities into binary scores (presence versus absence). The programme computes, for each clone, several quality parameters including (i) between-cluster variance in relative hybridization intensity as a percentage of the total variance (*P* value) (ii) the percentage of data points with defined allele calls (call rate), and (iii) the fraction of concordant calls for replicate assays (C. Cayla, G. Uszynski and A. Kilian, unpublished). This improved software version localized spots, rejected those with a weak reference signal, computed and normalized the relative hybridization intensities (=log(cy3target/cy5reference) or log(cy3target/FAMreference)), calculated the median value for replicate spots, identified polymorphic clones by using a combination of ANOVA and fuzzy C-means clustering at a fuzziness level of 1.5 and classified polymorphic clones as being present (1) or absent (0) in the representation hybridized to a slide. The clustering algorithm also provided a probability estimate for each individual genotype call (C. Cayla, G. Uszynski and A. Kilian, unpublished).

Polymorphism information content (PIC), a measure of informativeness of these DArT markers was calculated according to Anderson *et al.* (1993):

$$\text{PIC} = 1 - \sum_{i=1}^{k} P_i^2.$$

Where P_i is the population frequency of the *i*th allele and *n* the total number of allelic states. The segregation of each marker was estimated by applying chi-square (χ^2) test and probability was calculated.
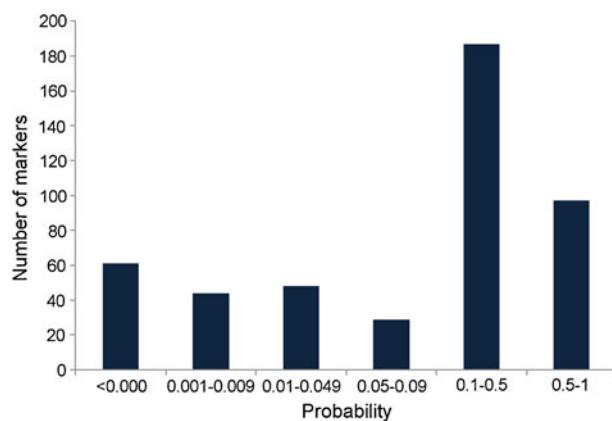
Linkage mapping

MAPMAKER/EXP version 3.0b software (Lander *et al.* 1987) was used to analyse the DArT markers to generate statistical information and mapping distances. Once the dataset was sorted, each group was used to check marker orders and obtain marker statistics. The exact order of the markers within each group was determined by using the 'try' command, which compares the maximum-likelihood of each marker order after keeping the markers, one by one, into every interval of the established order. Small sorting errors were removed using the 'Ripple' command on all groups. A Kosambi map function was used to convert recombination frequency into cM distances. The graphical maps of the linkage groups were constructed by using QTL Cartographer version 2.5 (Wang *et al.* 2007).

Results

Evaluation of DArT markers quality

Among 466 polymorphic (from a total of 554) DArT markers, 198 were ICP 28 (maternal) specific and 268 were ICPW 94 (paternal) specific. The majority of polymorphic DArT markers (284, 60.9%) showed expected segregation (i.e., 1 : 3 or 3 : 1, $P > 0.05$) (figure 1). The remaining polymorphic DArT markers (182, 39.1%) deviated from the expected ratio. Distorted markers showing probability higher than 0.001 were also tried for mapping in the framework linkage groups of both maternal and paternal maps. The segregating markers were classified into two classes; for DArT class 1 consensus marker the selection criterion were of very high stringency parameters with clustering settings; $Q > 70$; $P > 75$; call rate > 90, 100% reproducibility, no discordance, and probability > 0.001. A total of 345 consensus DArT markers were discovered by this selection and the average call rate of this class of DArT markers is 97.7; and their PIC values ranged from 0.047 to 0.497, with an average of 0.349 (see table 1 in electronic supplementary material at http://www.ias.ernet.in/jgenet/). The second class of DArT markers (DArT class 2) were selected using a slightly lower stringency and selection criteria parameters $Q > 70$; call rate > 80, 0.55–0.0 discordance, and probability > 0.001. By this process a further 43 DArT markers were selected. The average call rate of DArT markers of this class is 91.8; and their PIC values range from 0.364 to 0.495, with an average of 0.364. Their average reproducibility is 99.32 and discordance is 0.033 (see table 1 in electronic supplementary material). In summary, a total of 388 DArT markers (345 from the class 1 and 43 from the class 2) were selected for linkage mapping.

Figure 1. Distribution of segregation of 466 polymorphic DArT markers on 72 F_2s.

Table 2. Details on 466 polymorphic DArT markers.

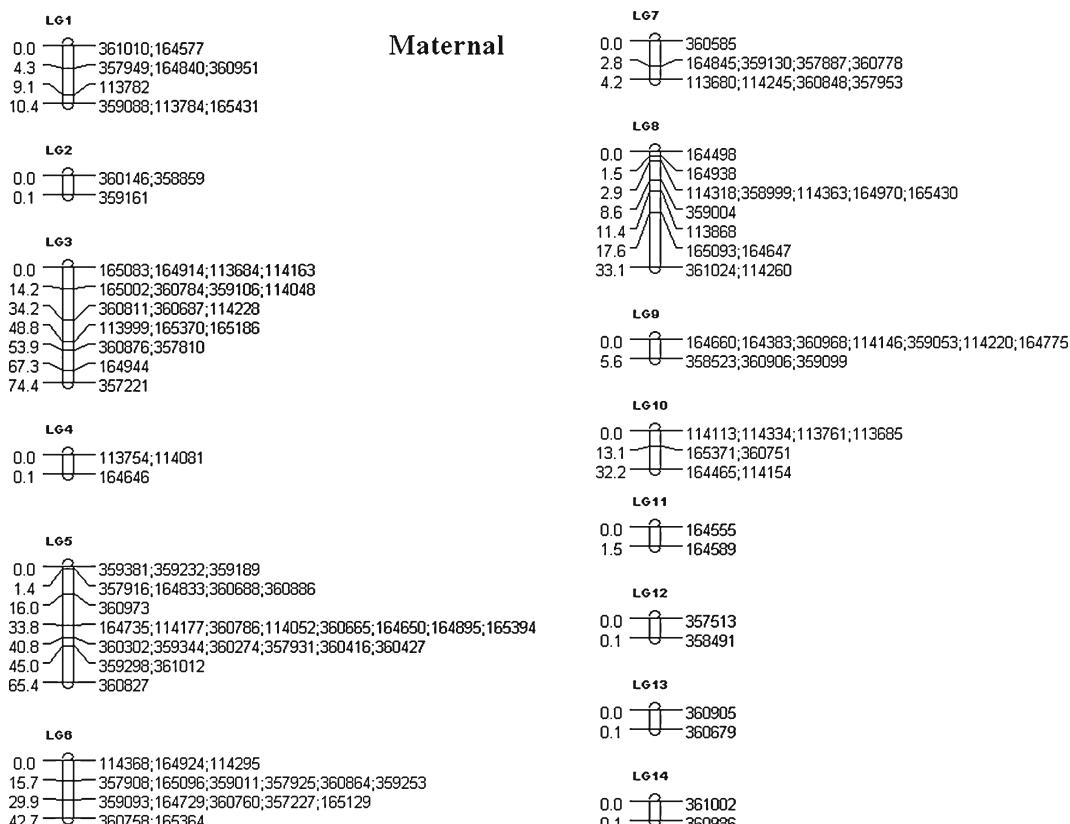|	Female specific	Male specific
Total	198	268
Class I	142	203
Class II	27	16
Others	29	49
Mapped	122	172

Linkage mapping

DArT experiments performed on the F_2 progenies of ICP 28 × ICPW 94 provided high quality data for 388 DArT markers for mapping which included 169 detected from maternal and 219 DArT markers detected from paternal parent (table 2). The binary scores of polymorphic markers were used to search the suitable mapping criterion to construct the framework genetic linkage map in pigeon pea. Different linkage criteria were tested for mapping and finally the markers were mapped at logarithm of odds (LOD) score of 5.0 and maximum recombination frequency of 20%. It was observed that at higher recombination frequency (> 20%), markers tend to cluster on one or two linkage groups.

The data of both sets of markers (maternal and paternal) was combined and also used to prepare a joint map of pigeon pea. However, the maternal and paternal markers occupied separate linkage groups. Therefore two separate maps were prepared in the present study. The maternal markers generated 14 linkage groups (figure 2). As a result 122 unique DArT maternal markers (72.18% of the total unique maternal markers) generated pigeon pea maternal linkage map with the total length of 270.0 cM and an average marker distance of 2.2 cM per marker. The total length of 14 linkage groups ranged from 0.1 cM to 74.4 cM (figure 2; table 3). With the same LOD value of 5.0, 16 linkage groups were generated for paternal markers (figure 3). A total of 172 unique DArT paternal markers (78.53% of the total unique paternal markers) generated pigeon pea paternal linkage map with the total length of 451.6 cM with average marker distance of 2.6 cM per marker. The total length of 16 linkage groups ranged from 0.1 cM to 157.9 cM (figure 3; table 3).

Discussion

Development of a framework linkage map is an important pre-requisite for identification of QTL(s) as well as

Figure 2. Pigeon pea linkage map using DArT maternal markers; name of markers are on the right side, while the cumulative genetic distances (cM) are shown on the left side of the map.

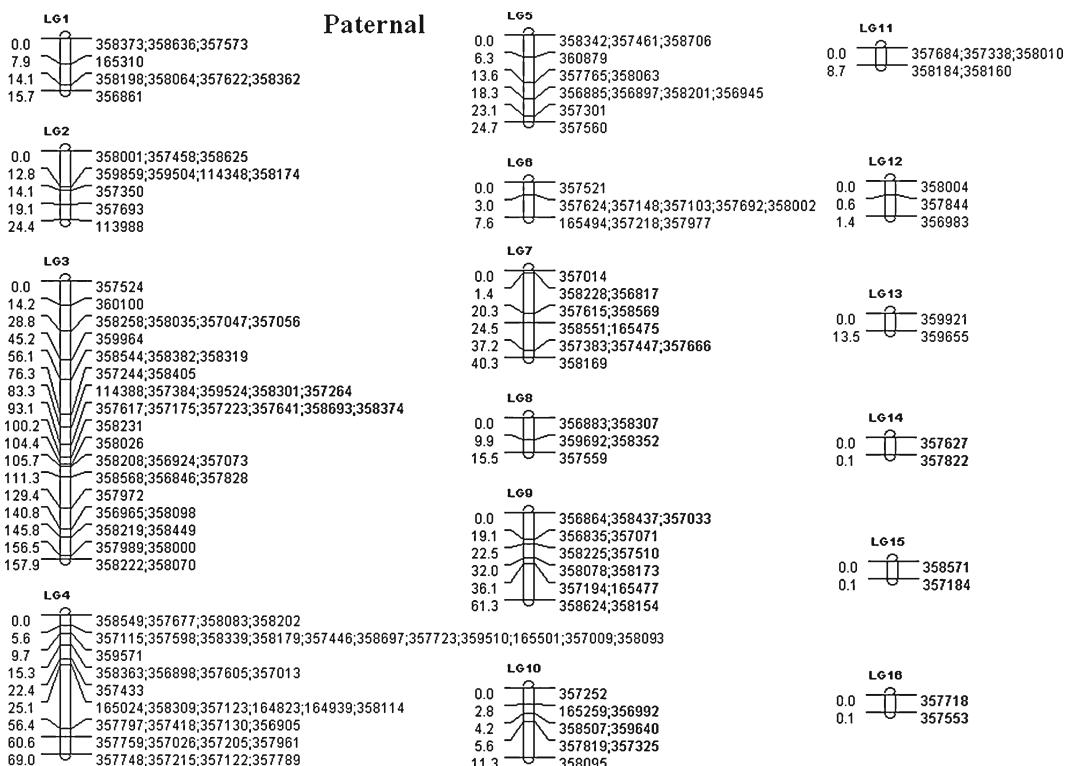**Table 3.** Characteristics of maternal and paternal maps of pigeon pea.

Linkage group	No. of markers		Length (cM)		Density (marker/cM)		No. of clusters	
	M	P	M	P	M	P	M	P
1	9	9	10.4	15.7	1.16	1.74	0	1
2	3	10	0.1	24.4	0.03	2.44	0	1
3	18	40	74.4	157.9	4.13	3.95	2	3
4	3	39	0.1	69.0	0.03	1.77	0	7
5	25	12	65.4	24.7	2.62	2.06	3	1
6	16	9	42.7	7.6	2.51	0.84	2	1
7	9	11	4.2	40.3	0.47	3.66	2	0
8	13	5	33.1	15.5	2.55	1.94	1	0
9	10	13	5.6	61.3	0.56	4.72	1	0
10	8	8	32.2	11.3	4.03	1.41	1	0
11	2	5	1.5	8.7	0.75	1.74	0	0
12	2	3	0.1	1.4	0.05	0.47	0	0
13	2	2	0.1	13.5	0.05	6.75	0	0
14	2	2	0.1	0.1	0.05	0.05	0	0
15	-	2	-	0.1	-	0.05	-	0
16	-	2	-	0.1	-	0.05	-	0
$\sum$/average	122/8.7	172/10.7	270	451.6	2.2	2.6	12	14

M, maternal; P, paternal.

accelerated marker-assisted selection in any crop improvement programme. One of the reasons for slow progress in the molecular breeding of the important legume crop like pigeon pea has been attributed to the limited availability of molecular markers and non-availability of any linkage map so far. Although, tremendous progress has been made in terms of availability of genomic resources in this orphan legume crop in recent times (Varshney *et al.* 2009, 2010), availability of a framework linkage map was lacking. In the present study, we report the first framework linkage map of pigeon pea based on an interspecific F_2 mapping population using DArT markers.

Figure 3. Pigeon pea linkage map using DArT paternal markers.

DArT was developed as a hybridization-based microarray platform technology which used rice as a model (Jaccoud *et al.* 2001). The important advantage of using these markers is that they can simultaneously type several hundreds to thousands loci in a single assay and provide a cost-effective and sequence-independent tool for whole-genome fingerprinting. DArT generates genetic fingerprints by scoring the presence versus absence of DNA fragments in genomic representations generated from samples of genomic DNA. Hence, DArT was rapidly applied to a number of other plants and animals such as: apple, *Arabidopsis thaliana*, banana, barley, cassava, chickpea, coconut, eucalyptus, several fungal pathogens, lupin, pigeon pea, potato, quinoa, rice, ryegrass, sorghum, tomato, mouse, sheep and cattle (Jaccoud *et al.* 2001; Lezar *et al.* 2004; Wenzl *et al.* 2004; Kilian *et al.* 2005; Wittenberg *et al.* 2005; Xia *et al.* 2005; Yang *et al.* 2006; Akbari *et al.* 2006; www.diversityarrays.com). The other advantage of DArT is the speed of development in a new species. Once a method and an array is developed, there are advantages in using DArTs compared to other marker technologies. The lower cost of genotyping with DArT is a major advantage compared to other newly developing technologies such as SNP. Considering these advantages, DArT technique was used to prepare first framework linkage map in the present study.

In the present study, certain number of markers did not conform to the expected segregation ratio and most of these distorted markers could not be mapped on the linkage maps. A number of reasons are responsible for segregation distortion such as rearrangement in genome, allele inducing gametic or zygotic selection (Lu *et al.* 2002), parental reproductive differences and the presence of lethal genes (Blanco *et al.* 1998). Use of wild relative in developing mapping population might be one possible cause of segregation distortion of markers in the present study. As expected, majority of mapped DArT markers (94.2% and 98.2% in maternal and paternal maps, respectively) were showing probability of segregation higher than 0.05 and only seven DArT markers with distorted segregation, i.e. $P < 0.05$ could be mapped in maternal map whereas, in case of paternal map only three DArT markers with distorted segregation i.e., $P < 0.05$ could be integrated.

As compared to the co-dominant markers like SSRs, it was observed that a considerable number of DArT markers were clustered (more than three markers at the same position) in specific regions of different linkage groups of both the maps. The clustering of the DArT markers indicated the presence of 12 and 14 clusters in maternal and paternal maps, respectively (table 3). The maximum numbers of clusters were 3 in linkage group 5 of maternal map, while in case of paternal map there were 7 clusters on linkage group 4 (table 3). Markers sometimes tend to cluster because of uneven distribution of recombination events (Tanksley *et al.* 1992) or due to unequal survey of DNA polymorphism that is unevenly distributed along genome (Liang *et al.* 2002). In some linkage groups, clustering appeared to be higher in central and distal regions. This pattern may reflect a moderate bias of *Pst*I-based DArT markers towards gene-rich hypomethylated areas in the genome (Moore 2000). Such sort of clustering using dominant markers is not uncommon. For example, clustering of markers in the centromeric regions of the chromosomes has also been reported by Sandal *et al.* (2002) and Saal and Wricke (2008) in an F_2 mapping population in lotus and rye, respectively using AFLP markers.

The mapping population of pigeon pea used in the present study was generated from a cross between cultivated (*C. cajan*) genotype and its wild relative (*C. scarabaeoides*). As genetic diversity in cultivated pigeon pea (*C. cajan*) is low (Burns *et al.* 2001; Yang *et al.* 2006), linkage maps have not been constructed in intraspecific mapping populations so far. Sufficiently large numbers of common markers are required possibly with good SSRs or some other co-dominant markers to establish synteny/colinearity between these maps in future. However, the number of markers developed in the present study could prove to be an useful genomic resource for interspecific genetic analysis in pigeon pea. In addition, availability of the first linkage map will allow map-based cloning of genes from wild relatives in future, such as genes for biotic and abiotic stress tolerance/resistance in pigeon pea. This will be facilitated particularly with the utilization of a large number of SSR and SNP markers that will be available soon in public domain in this crop (Varshney *et al.* 2010).

Although pigeon pea has 11 chromosomes, all the polymorphic makers fell into 14 maternal and 16 paternal linkage groups. We expect that the small linkage groups will be merged into larger ones with the availability of additional set of markers. Complete description of the linkage groups with pigeon pea chromosomes, however would be difficult to get with an interspecific mapping population alone due to incompleteness of the homologous recombination (Livingstone *et al.* 1999). Moreover, direct comparison of these linkage maps with other legume maps is not possible at the moment because of unavailability of maps in legume species which are developed by using this strategy. Similar approaches (maternal and paternal maps) in mapping with dominant class of markers have also been used in tetraploid willows (Barcaccia *et al.* 2003), guinea yam (Mignouna *et al.* 2002), and dioecious texas bluegrass (Renganayaki *et al.* 2005). The present study represents the first genetic linkage map for pigeon pea, and is the first of its kind using DArT markers in any grain legume crop. The most immediate application of the molecular linkage maps will be locating markers linked to genes of economic interest (e.g. *Helicoverpa* resistance) and aligning future genetic maps with it.

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